Bioassays of resistance of transgenic poplar with novel binary insect-resistant genes to *Anoplophora glabripennis* (Coleoptera: Cerambycidae) and *Hyphantria cunea* (Lepidoptera: Arctiidae)

YANG Li-Yan[1,2,3], SUN Yi[1,*], XIE Li-Qin[1]

(1. Biotechnology Institute, Shanxi University, Taiyuan 030006, China;
2. Agri-Biotechnology Research Centre of Shanxi Province, Taiyuan 030031, China;
3. Shanxi Normal University, Linfen, Shanxi 041000, China)

Abstract: To verify the resistance of binary genes chitinase-BmkIT to larvae of Coleopterous insect *Anoplophora glabripennis* and Lepidopterous insect *Hyphantria cunea*, *in vitro* insect-resistant bioassays were conducted, where the 1st-instar larvae of *A. glabripennis* were inoculated into poplar stems and those of *H. cunea* onto fresh poplar leaves. The results showed that transgenic clone 132-2, 144-1 and 123-1 had significant lethal and inhibitory effects on *H. cuneal* larvae compared with the untransformed control ($P < 0.05$). Most *H. cunea* larvae were not able to develop into pupae, some pupae were not able to emerge, and some emerged moths were abnormal with imperfect wings which died soon without mating. However, the differences in larval mortality and body weight of *A. glabripennis* were not significant compared with those of the untransformed control, indicating that transgenic stems had little influence on the survival and development of *A. glabripennis* larvae. The results suggested that the insect-resistant gene combination could not only be used as one of the complementary alien gene sources for resisting Lepidopterous pests but also contributed to reducing the risk of development of insect resistance produced by using single insect toxin gene.

Key words: Transgenic poplar; chitinase gene; BmkIT gene; *Hyphantria cunea*; *Anoplophora glabripennis*; insect resistance

1 INTRODUCTION

Poplars are the most important pulpwood material and potential bioenergy tree species in the world. The completed poplar genome database (Tuskan *et al.*, 2006) has made them the most useful model species for studying molecular biology of woody plants. In North China, poplar are highly effective pioneer species, but the widely-distributed man-made poplar forests are venerable to attacks of various insect pests, and more than 100 000 hm^2 of poplar is devastated by various pests per year, which causes economic loss of 100 million RMB (Lu and Hu, 2006).

The Asian long-horn beetle *Anoplophora glabripennis* is a severe Coleopterous insect on poplar which causes fatal damages in nature shelters in North China (Zhang *et al.*, 2005) and there is no effective way to control its infestation up to now. The fall webworm *Hyphantria cunea* is an invasive Lepidopterous insect pest in China, and this webworm defoliates ornamental, forest and fruit trees as well as agricultural crops. Because of the severe damages the fall webworm wreaks on its host plants, pesticides were commonly applied but they proved to be less than ideal.

Genetic modification has the potential to contribute to improving plant resistance to insects. At present, *Bacillus thuringiensis* (*Bt*) gene is the most effective and widely used insect toxin gene in poplar genetic engineering (Tian *et al.*, 2000; Massimo *et al.*, 2001; Zhang *et al.*, 2005; Lin *et al.*, 2006). However, the emergence of insect strains that evolved Bt-resistance has brought about more and more concerns (McGaughey, 1985; Jin *et al.*, 2000; Tabashnik *et al.*, 2006). Developing novel insect-resistant genes is a desirable pest control management.

We transferred a binary gene construct (Zhang *et al.*, 2004) that contains the chitinase gene from

基金项目: 山西省科技攻关项目(2006031015)
作者简介: 杨利艳, 女, 1972 年生, 博士研究生, 从事生物化学和分子生物学研究, E-mail: liyanyang1972@yahoo.com.cn
* 通讯作者 Author for correspondence, E-mail: sunyi692003@yahoo.com.cn
收稿日期 Received: 2008-04-11; 接受日期 Accepted: 2008-06-27

Manduca sexta and the Bmk gene encoding a scorpion insect toxin (BmkIT) from *Buthus martensii* Karsch into a poplar cultivar, Zhonglinmeihe. Chitinase is an enzyme capable of hydrolyzing insoluble chitin to its oligo and monomeric components, while Bmk is a scorpion neurotoxin of contractive paralysis type which has none or little harm to mammals and acts only on insects (Barton, 1990). Here we report the results of the insect-resistant bioassays of the transgenic poplar clones using *A. glabripennis* larvae and *H. cunea* larvae.

2 MATERIALS AND METHODS

2.1 Plant material and tested insects

Transgenic poplars harboring chitinase-BmkIT genes are provided by Agri-Biotechnology Research Centre of Shanxi Province.

A. glabripennis 1st-instar larvae were provided by the Research Institute of Forest Ecology, Environment and Protection (RIFEEP), Chinese Academy of Forestry, Beijing, China; *H. cunea* eggs were collected from Qinhuangdao, Hebei, China. The insect eggs were collected in a bottle that was covered with cloth to ensure ventilation and with some fresh leaves in it to keep humidity, under a constant temperature of 26 ± 1℃. Neonates two days after hatching were carefully transferred to tested stems or leaves by a soft brush to conduct bioassay.

2.2 Bioassay methods

2.2.1 Bioassay of *A. glabripennis* larvae: Transgenic (132-2, 144-1, 123-1, 178-2, 43-42, 43-47, 20-10, 43-39) and untransformed control branches (CK) with diameters of above 2 cm were sawed into segments of about 20 cm. In each segment, three tunnels were dug with their barks attached on the branches and one larva was put into each tunnel with barks covered back and then tunnels were wrapped with parafilm, respectively. Two ends of each segment were wrapped with wet cotton wools and parafilm which were kept wet with periodical watering to prevent dehydration. Poplar segments with inoculated larvae were maintained at a constant temperature of 25 ± 1℃ under 16L∶8D photoperiod. Each poplar clone was replicated for 3 times, and larval mortality and weight as well as length of the tunnel caused by the larvae were recorded a month later.

2.2.2 Bioassay of *H. cunea* larvae: Leaves from transgenic poplar clones (132-2, 144-1, 123-1, 178-2) and the untransformed control were placed in petri dishes (9 cm in diameter) on a piece of wet filter paper, 15 neonates two days after hatching were plated per petri dish which was sealed with parafilm to prevent the escape of larvae. Tested leaves with inoculated larvae were maintained at a constant temperature of 25 ± 1℃ under 16L∶8D photoperiod. Each treatment was replicated for 3 times, and larval mortality, weight and body length were recorded every two days.

2.3 Statistics analysis

SPSS10.0 program was adapted, statistical significances among treatments were determined by one way ANOVA, and Pearson correlation coefficient was calculated between larva body weight and tunnel length caused by larva in stem.

3 RESULTS

3.1 Bioassay of *A. glabripennis* larvae on transgenic stems

Mortalities of *A. glabripennis* larvae were recorded a month later. Statistical analysis revealed that, compared with the untransformed control, larval mortalities fed on transgenic clones 43-42, 43-47 and 43-39 were higher, that on 20-10 was almost the same, whereas those on 132-2, 144-1, 123-1, 178-2 were lower (Table 1). However, the differences were insignificant between those fed on transgenic stems and the untransformed control ($P > 0.05$), which indicated that the transgenic stems did not have significant impact on survival of *A. glabripennis* larvae. Larva body weight increases could be used to measure the larval growth and development situations, which were indicators of effects caused by transgenic stems. The longest tunnel was in clone 20-10 where increased body weight of larva was also the biggest. Length of the tunnel dug by *A. glabripennis* larvae was correlated significantly with larval body mass ($r = 0.816^{**}$), which means inhibition of larval growth can decrease the damage, but differences of tunnel length and larval mass were both insignificant ($P > 0.05$) between those on transgenic stems and the untransformed control.

3.2 Bioassay of *H. cunea* larvae on transgenic poplar leaves

The development processes of *H. cunea* larvae fed on transgenic and untransformed control leaves were

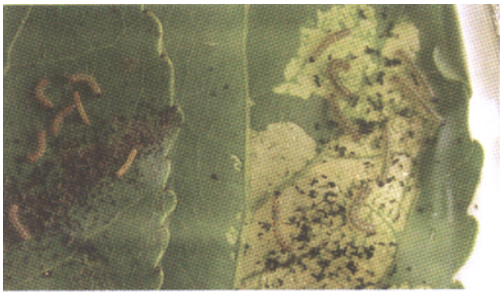

Fig. 1 The damage area of transgenic leaf (left) *vs* the untransformed control (right)

recorded. Overall, during 14 days, the mortalities of larvae fed on transgenic leaves were consistently greater than those on control leaves (Table 2). Within them, the mortalities on clones 123-1, 144-1 and 132-2 were significantly higher than on control for the whole duration ($P < 0.05$), but differences between that on 178-2 and control was insignificant ($P > 0.05$). The larval weight and body length on transgenic leaves were also consistently lower than that on the untransformed control, and the differences were statistically significant from 8th day on ($P < 0.05$) except on 178-2 (Table 3 and 4). Accordingly, less feeding damages were observed (Fig. 1) on transgenic leaves.

Table 1 Results of inoculating *Anoplophora glabripennis* larvae

Poplar clone no.	Average body weight before inoculation (g)	Average mortality (%)	Average body weight 30 days later (g)	Increased body weight rate (%)	Average tunnel length (cm)
CK	0.047	22.2 ± 19.22 a	0.232 ± 0.027 a	393.6	6.38 a
132-2	0.049	11.1 ± 19.22 a	0.253 ± 0.079 a	416.3	6.84 a
144-1	0.048	11.1 ± 19.22 a	0.208 ± 0.055 a	333.3	6.26 a
123-1	0.050	11.1 ± 19.22 a	0.251 ± 0.15 a	402.0	7.02 a
178-2	0.047	11.1 ± 19.22 a	0.207 ± 0.04 a	340.4	6.89 a
43-42	0.048	44.4 ± 19.28 a	0.211 ± 0.035 a	339.6	6.21 a
43-47	0.051	33.3 ± 19.22 a	0.229 ± 0.069 a	349.0	6.28 a
20-10	0.046	22.2 ± 19.22 a	0.295 ± 0.043 a	541.3	7.85 a
43-39	0.052	44.4 ± 19.28 a	0.168 ± 0.041 a	223.1	6.19 a

Notes: Data in the table are means ± *SD* (n = 3) or means of 3 replications, and those in the same column followed by different letters were significantly different at 5% level by Duncan's multiple range test. The same below. Increased body weight rate (%) = (body mass 30 days later − body mass before inoculation)/body mass before inoculation × 100%

Table 2 Mortality (%) of *Hyphantria cunea* larvae fed on transgenic poplar leaves for different days

Poplar clone no.	Feeding days					
	4 d	6 d	8 d	10 d	12 d	14 d
CK	4.0 ± 3.46 a	8.3 ± 4.04 a	10.7 ± 4.04 a	10.7 ± 4.04 a	26.3 ± 11.55 a	26.3 ± 11.55 a
132-2	28.9 ± 1.41 b	28.9 ± 15.41 b	31.1 ± 19.23 bc	39.9 ± 11.55 b	42.2 ± 15.41 a	57.8 ± 7.74 b
144-1	28.9 ± 3.81 b	28.9 ± 3.81 b	33.3 ± 6.65 bc	48.9 ± 3.81 bc	62.2 ± 3.87 b	77.8 ± 19.23 b
123-1	24.5 ± 3.87 b	37.8 ± 3.87 b	46.7 ± 6.65 c	57.8 ± 3.87 c	62.2 ± 7.74 b	77.8 ± 19.23 b
178-2	4.5 ± 3.87 a	13.3 ± 6.65 a	24.5 ± 3.87 ab	37.8 ± 3.87 b	44.5 ± 3.87 ab	53.3 ± 6.65 b

Table 3 Body mass (g) of *Hyphantria cunea* larvae fed on transgenic poplar leaves for different days

Poplar clone no.	Feeding days			
	8 d	10 d	12 d	14 d
CK	0.0130 ± 0.002 a	0.0220 ± 0.0013 a	0.0320 ± 0.002 a	0.0530 ± 0.0047 a
132-2	0.0027 ± 0.0006 c	0.0047 ± 0.0015 c	0.0117 ± 0.0015 c	0.0207 ± 0.0025 a
144-1	0.0043 ± 0.0006 c	0.011 ± 0.0015 b	0.0117 ± 0.0006 c	0.0357 ± 0.0031 a
123-1	0.0023 ± 0.0006 c	0.0037 ± 0.0012 c	0.0093 ± 0.0006 c	0.0390 ± 0.044 a
178-2	0.0067 ± 0.0012 b	0.0200 ± 0.0015 a	0.0127 ± 0.0012 a	0.0403 ± 0.004 a

Table 4 Body length (cm) of *Hyphantria cunea* larvae fed on transgenic poplar leaves for different days

Poplar clone no.	Feeding days			
	8 d	10 d	12 d	14 d
CK	0.9333 ± 0.12 a	1.4330 ± 0.20 a	1.4333 ± 0.21 a	1.7333 ± 0.21 a
132-2	0.6436 ± 0.31 c	0.9750 ± 0.46 c	1.1052 ± 0.42 cd	1.3302 ± 0.52 b
144-1	0.6333 ± 0.06 b	0.9667 ± 0.06 b	1.2333 ± 0.06 ab	1.5667 ± 0.058 a
123-1	0.5333 ± 0.06 bc	0.6667 ± 0.12 c	0.8667 ± 0.06 d	1.7333 ± 0.058 a
178-2	0.6667 ± 0.06 b	0.9667 ± 0.06 b	1.1667 ± 0.06 bc	1.4667 ± 0.25 ab

Table 5 Effects of transgenic poplar leaves on *Hyphantria cunea* progeny

Shoots no.	Pupation rate (%)	Eclosion rate (%)	Abnormal eclosion rate (%)
CK	33.3 ± 9.78	60 ± 20.00	0
132-2	33.3 ± 8.44	60 ± 17.32	66.7 ± 15.33
144-1	0	0	0
123-1	6.67 ± 2.66	0	
178-2	0		

Not only could the leaves from transgenic poplar lines enhance mortality of *H. cunea* larvae but also inhibit the development of survived larvae, causing reduced body weight and length. Furthermore, the reproduction of the pest was interfered and their progenies were abnormal and weak (Table 5). Among the tested transgenic clones, we observed larvae reared on 144-1 and 178-2 could not develop into pupae though their body became dark and sluggish (Fig. 2: a); larvae fed on 123-1 could develop into pupae, but the pupae could not emerge; larvae on leaves of clone 132-2 could emerge to abnormal moths with imperfect wings, which died soon (Fig. 2: b) without mating.

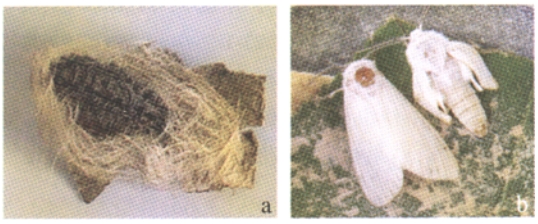

Fig. 2 Effects of transgenic leaves on *Hyphantria cunea* progeny
a: Larvae could not develop into pupae; b: Normal eclosion (left) *vs* abnormal eclosion with imperfect wings (right).

4 CONCLUSION

H. cunea is one of the most serious invasive species in China, which is native to North America and was first found in Liaoning Province, China in 1979. At present, it has spread to Beijing, Tianjin, Hebei, Liaoning, Shandong and Shanxi, *etc*. Not only does *H. cunea* cause serious leaf damage throughout its infestation range and appears to be continuing to spread, but in a long term, invasion of alien species is one of the important factors endangering global biodiversity, which reduces the uniqueness of regional fauna and flora, and breaks down geographical barriers that maintain global biodiversity.

Wang *et al*. (2005) firstly introduced the insect-resistant gene combination, chitinase gene and scorpion insect toxin gene, into a *Brassica napus* cultivar and the transgenic rapeseed plants showed resistance against larval infestation of diamondback moth (*Plutella maculipenis*). In the present study, transgenic poplar leaves showed resistance against *H. cunea* larval infestation in that larval mortality were higher and their development were inhibited. Our results suggested that the insect-resistant gene combination could be used as a new pest resistance gene source which might be one of the complementary alien gene sources to *Bt* toxin gene to broaden the insecticidal spectrum and to reduce the risk of development of insect resistance produced by using single insect toxin gene. Moreover, our study manifested that the binary insect resistant gene could not significantly inhibit the growth of *A. glabripennis* larval infestation. The results also indicated that the binary gene construct was useful for controlling Lepidopterous but not Coleopterous insects. Therefore, we need to search for other gene sources for combating Coleopterous pests' infestation.

ACKNOWLEDGEMENTS The authors would like to thank Profs. Zhang Yong-An and Wang Yu-Zhu of Research Institute of Forest Ecology, Environment and Protection (RIFEEP), Chinese Academy of Forestry, Beijing for providing *A. glabripennis* larvae and their technical assistances.

References

Barton KA, 1990. Insecticidal toxins in plants-express an insect-specific toxin from a scorpion. European Patent [Patent No. EP0431829A1].

Jin RG, Liu YB, Tabashnik BE, Borthakur D, 2000. Development of transgenic cabbage (*Brassica oleracea* var. *capitata*) for insect resistance by *Agrobacterium tumefaciens*-mediated transformation. *In Vitro Cellular and Developmental Biology Plant*, 36: 231 – 237.

Lin T, Wang ZY, LIU KY, Jing TZ, 2006. Transformation of spider neurotoxin gene with prospective insecticidal properties into hybrid poplar *Populus simonii* × *P. nigra*. *Acta Entomologica Sinica*, 49 (4): 593 – 598.

Lu MZ, Hu JJ, 2006. Study on transgenic poplar and its utilization in China. *China Forestry Science and Technology*, 20(6): 1 – 4. [卢孟柱, 胡建军, 2006. 我国转基因杨树的研究及应用现状. 林业科技开发, 20(6): 1 – 4]

Massimo D, Cianni A, Beatrice B, Alma B, Franco P, Alex L, Samantha Z, Paolo C, Massimo C, 2001. Transformation of white poplar (*Populus alba* L.) with a novel *Arabidopsis thaliana* cysteine proteinase inhibitor and analysis of insect pest resistance. *Molecular Breeding*, 7: 35 – 42.

McGaughey WH, 1985. Insect resistance to the biological insecticide *Bacillus thuringiensis*. *Science*, 229: 193 – 194.

Tabashnik BE, Biggs RW, Fabrick JA, Gassmann AJ, Dennehy TJ, Carrière Y, Morin S, 2006. High-level resistance to *Bacillus thuringiensis* toxin crylac and cadherin genotype in pink bollworm. *J. Econ. Entomol.*, 99 (6): 2 125 – 2 131.

Tian YC, Zheng JB, Yu HM, Liang HY, Li CQ, Wang JM, 2000. Selection and insect resistance of transgenic hybrid poplar 741 carrying two insect-resistant genes. *Scientia Silvae Sinicae*, 36 (2): 13 – 19. [田颖川, 郑均宝, 虞红梅, 梁海永, 李常青, 王进茂, 2000. 转双抗虫基因杂种 741 毛白杨的研究, 植物学报, 36(2): 13 – 19]

Tuskan GA, DiFazio S, Jansson S, *et al*., 2006. The genome of black

cottonwood, *Populus trichocarpa* (Torr & Gray). *Science*, 313 (5 793): 1 596 – 1 604.

Wang JX, Chen Zl, Du JZ, Sun Y, Liang AH, 2005. Novel insect resistance in *Brassica napus* developed by transformation of chitinase and scorpion toxin genes, *Plant Cell Rep*., 24: 549 – 555.

Zhang BY, Su XH, Li YL, Zhang YA, Qu LJ, Wang YZ, Tian YC, 2005. Transformation of poplar with binary insect resistant genes and analysis of insect resistance. *Forest Research*, 18(3): 364 – 368. [张冰玉, 苏晓华, 李义良, 张永安, 曲良建, 王玉珠, 田颖川, 2005. 转双价抗蛀干害虫基因杨树的获得及其抗虫性鉴定. 林业科学研究, 18(3): 364 – 368]

Zhang ZY, Zhang HS, SY, Liang AH, 2004. Construction of plant expression vectors containing two anti-insect genes. *Acta Botanica Boreali-occidentalia Sinica*, 24 (8): 1 402 – 1 408. [张志云, 张虎生, 孙毅, 梁爱华, 2004. 双价抗虫基因植物表达载体的构建. 西北植物学报, 24 (8): 1 402 – 1 408]

(责任编辑: 袁德成)

转新型双价抗虫基因杨树对光肩星天牛及美国白蛾的抗性鉴定

杨利艳[1,2,3], 孙 毅[1,*], 谢莉琴[1]

(1. 山西大学生物技术研究所, 太原 030006; 2. 山西省农业生物技术研究中心, 太原 030031;
3. 山西师范大学生命科学学院, 山西临汾 041000)

摘要: 为明确双价基因 chitinase-BmkIT 对鞘翅目害虫光肩星天牛及鳞翅目害虫美国白蛾的抗虫效果, 分别以转该双价基因的杨树茎干和叶片为材料, 采用室内茎干接虫和叶片接虫方法鉴定其对光肩星天牛 *Anoplophora glabripennis* 和美国白蛾 *Hyphantria cunea* 幼虫的抗性。结果表明: 转基因株系 132-2, 144-1, 123-1 对美国白蛾幼虫的致死作用和生长发育影响显著高于取食未转化叶片的处理($P < 0.05$), 且对其后代产生了影响, 表现为幼虫不能化蛹或化蛹后不能羽化或羽化不正常; 但供试的 8 个转基因株系对光肩星天牛幼虫无明显的致死和生长抑制作用, 表现为幼虫死亡率和体重均与取食非转化茎干幼虫无显著差异($P > 0.05$)。结果提示, 双价基因 chitinase-BmkIT 可作为杨树鳞翅目害虫的抗虫基因源, 并且对延缓害虫抗性的产生具有一定的意义。

关键词: 转基因杨树; 几丁质酶基因; 蝎昆虫特异性神经毒素基因; 美国白蛾; 光肩星天牛; 抗虫性

中图分类号: Q943.2 **文献标识码**: A **文章编号**: 0454-6296(2008)08-0844-05